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Redox-responsive in vitro modulation of the signalling state of the isolated PrrB sensor kinase of *Rhodobacter sphaeroides* NCIB 8253

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Abstract **Prr is a global regulatory system that controls a large and diverse range of genes in *Rhodobacter sphaeroides* in response to changing conditions of environmental redox potential. PrrB is the membrane-bound sensor kinase and previously we showed that the purified, detergent-solubilised intact membrane protein is functional in autophosphorylation, phosphotransfer and phosphatase activities. Here we confirm that it also senses and responds directly to its environmental signal, redox potential; strong autophosphorylation of PrrB occurred in response to dithiothreitol (DTT)-induced reducing conditions (and levels increased in response to a wide 0.1–100 mM DTT range), whilst under oxidising conditions, PrrB exhibited low, just detectable levels of autophosphorylation. The clear response of PrrB to changes in reducing conditions confirmed its suitability for in vitro studies to identify modulators of its phosphorylation signalling state, and was used here to investigate whether PrrB might sense more than one redox-related signal, such as signals of cell energy status. NADH, ATP and AMP were found to exert no detectable effect on maintenance of the PrrB–P signalling state. By contrast, adenosine diphosphate produced a very strong increase in PrrB–P dephosphorylation rate, presumably through the back-conversion of PrrB–P to PrrB.**

Keywords: PrrB; RegB; Redox potential; Phosphorylation; Two-component signal transduction

1. Introduction

The Prr signalling pathway of *Rhodobacter sphaeroides* (also previously known as Reg [1,2]) is a global transcriptional regulatory system that controls expression of a large number of genes in response to changes in redox potential [1,3,4]. Homologues occur in several photosynthetic and nitrogen fixing members of the α-proteobacteria [5–9], and one member of the γ-proteobacteria – the pathogen *Pseudomonas aeruginosa* [10]. PrrB is the membrane-located histidine protein kinase component of the pathway, sensing changes in redox potential conditions. Upon anaerobiosis, PrrB becomes phosphorylated at His-220 in an adenosine triphosphate (ATP)-dependent reaction, and PrrB–P then transfers the phosphoryl signal to Asp-63 of cognate response regulator PrrA [2]. Once phosphorylated, PrrA–P then positively or negatively regulates a wide range of genes and operons, which in *Rhodobacter* species include those involved in photosynthesis [1,3,5], carbon dioxide fixation [11,12], nitrogen fixation [13,14], electron transport and terminal oxidase/reductase functions [3,15,16] and hydrogenase activity [14]. PrrA is a two-domain protein, and the solution structure of the conserved C-terminal domain involved in DNA binding has been solved [17]. Alignment of DNA sequences recognised by PrrA (and RegA of *Rhodobacter capsulatus*) have revealed a consensus sequence for DNA binding [4,17]. The systems in *R. sphaeroides* and *R. capsulatus* have been reviewed [18,19].

The precise mechanism by which PrrB senses its redox signal(s) remains incomplete, though it is known to be connected to electron flow through the terminal cbb_3-type cytochrome *c* oxidase (Cco) of the electron transport chain [20]. Indeed, recent studies reveal that this complex directly regulates the phosphatase activity of PrrB towards PrrA, but not the autophosphorylation or kinase modes of the protein [21]. Presumably the transmembrane region of PrrB is involved, since our own studies and others have shown that this region is important for signalling [2,21]. In the closely related homologue RegB, a less direct role for Cco is proposed, involving signals from the ubiquinone pool [22]. However in addition, a redox-active cysteine located intracellularly in the soluble domain of RegB has also been implicated in controlling the signalling state [23]; under oxidising conditions RegB would form intermolecular disulfide bonds that convert RegB from an active signalling dimer into an inactive tetramer [23]. Although it cannot be ruled out that PrrB and RegB sense different redox signals, one possibility is that both proteins possess more than one 'input' for redox or redox-related signals – from electron transport and from the cytosol. Multiple modulators of two-component system signalling states have been reported previously e.g. [24,25]; in the case of redox sensor kinase ArcB in *E. coli*, cytosolic metabolites such as D-lactate, acetate, pyru-

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Abbreviations: PrrB, the intact membrane-bound sensor kinase (histidine protein kinase) from *Rhodobacter sphaeroides*; PrrA, the response regulator of the Prr two-component signal transduction system from *R. sphaeroides*; DTT, dithiothreitol; TCEP, tris[carboxyethyl]phosphine; PEP, phosphoenolpyruvate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Cco, cbb_3-type cytochrome *c* oxidase; DDM, *n*-dodecyl-β-D-maltoside

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vate and NADH (indirect indicators of redox state and energy status) were also found to play a role in modulating phosphorylation levels in addition to quinones of the electron transport chain [26].

Here we investigate factors linked to cell energy status (ATP, adenosine diphosphate (ADP), NADH) that could potentially modulate the signalling state of PrrB in response to changes in redox potential. We first show that our purified detergent-solubilised intact protein responds to redox signals as expected, and then that only ADP exerted an effect on PrrB–P through an effect on its dephosphorylation rate, presumably through the reversible conversion of PrrB–P to PrrB.

2. Materials and methods

2.1. Bacterial strains and plasmids, reagents and chemicals

Bacterial strains and plasmids have been described previously [2]. Restriction enzymes and phage T4 ligase were obtained from GibcoBRL; *Pfu* polymerase was obtained from Boehringer Mannheim. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from ICN Pharmaceuticals Ltd. DDM was obtained from Melford Biosciences. Agarose-immobilised Ni^{2+}-nitrilotriacetic acid (NTA) resin and anti-RGS(H_6) monoclonal antibody were obtained from Qiagen. Tris[carboxyethyl]phosphine (TCEP) was obtained from Molecular Probes, NL. All media, stock buffers and procedures for growth of bacterial cultures and DNA manipulations followed previous methods [27]. All other chemicals and reagents employed were of Analar or equivalent grade unless otherwise stated.

2.2. Purification of Prr proteins

Protocols have been described previously for the overexpression and purification of intact PrrB using *E. coli* NM554/pTTQ*regB* [2,28]. PrrA was expressed in *E. coli* BL21[DE3] harbouring pET*regA* as described [2].

2.3. In vitro phosphorylation assays

Assays were performed as detailed in [2]. Assays were initiated through the addition of 10 μl of radiolabelled ATP (10 μmol ATP containing 50 μCi of [γ-^{32}P]ATP or [γ-^{33}P]ATP). Samples (10–20 μl) were removed at intervals and reactions stopped by addition of 5 μl of 4× loading buffer [2]. If samples were not loaded immediately onto SDS–polyacrylamide gels, then they were stored at −70 °C.

2.4. ATP regenerative system

ADP was removed from phosphorylation assays using an ATP regenerative system routinely comprising 2 U pyruvate kinase and 1 mM phosphoenolpyruvate (PEP) per assay reaction [29].

3. Results

3.1. Intact PrrB autophosphorylates in response to reducing conditions induced by thiol exchanger dithiothreitol (DTT)

Previously, we noted that levels of in vitro PrrB phosphorylation could be significantly increased through addition of reducing agent DTT [2]. We investigated this further by examining the effects of various concentrations of DTT between 0 and 100 mM and the results are shown in Fig. 1. As the concentration of DTT present in the assays was increased, so too were the resulting levels of PrrB–P. This confirms that our intact purified protein here is functioning appropriately in response to reducing conditions. Interestingly, increases in PrrB–P were observed in the high 10–100 mM DTT range. This was unexpected, since this range presumably exceeds the concentrations required to achieve fully reduced conditions. One possibility is that trace amounts of nickel (potentially arising from protein purification) could reduce the activity of DTT in these assays, as reported previously [30]. Alternatively, supramillimolar concentrations of DTT (up to 150 mM DTT), similar to those used here were recently reported to extract some metals from proteins [31]; if nickel inhibits PrrB phosphorylation activity, then this inhibitory nickel might be progressively removed under these high DTT concentrations, resulting in increased PrrB activity [31]. Fig. 1 also shows very low, barely detectable levels of PrrB phosphorylation under air-oxidising conditions in the absence of DTT. The amount is significantly lower than that observed using just 0.1 mM DTT. Therefore, the purified PrrB used in these experiments, and as described in [2], is exhibiting clear-cut responses to oxidising as well as reducing conditions, and was therefore used in our subsequent experiments described below.

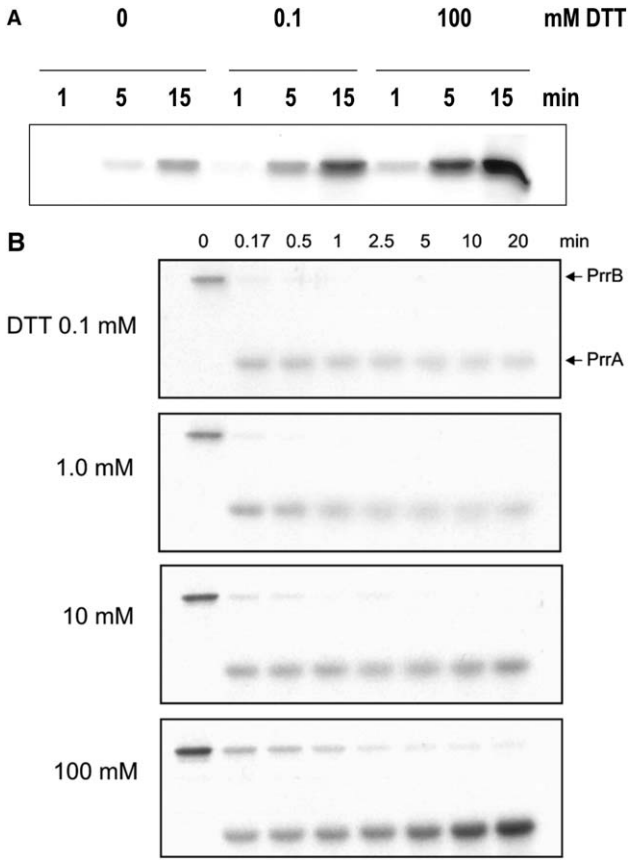

Fig. 1. Modulation of PrrB autophosphorylation and phosphotransfer to PrrA by dithiothreitol in vitro. (A) Effect of DTT on PrrB autophosphorylation. Autophosphorylation assays containing 172 pmol purified PrrB were initiated using 50 μM ATP and [γ-^{32}P]ATP in a total volume of 25 μl as described [2] in the presence of 0, 0.1 or 100 mM DTT. (B) Effect of DTT on PrrB autophosphorylation and subsequent phosphotransfer to PrrA. About 516 pmol PrrB was allowed to autophosphorylate as described above in the presence of 0.1, 1, 10 or 100 mM DTT for 20 min in a total volume of 170 μl. After withdrawal of 20 μl (time 0 sample), 1166 pmol purified PrrA were added to give a final PrrB:PrrA ratio of 1:2.5. About 20 μl samples were withdrawn at the times indicated. In all experiments, samples were removed to loading (stop) buffer, and resolved using 12% SDS–polyacrylamide gels. The quantity of ^{32}P associated with PrrB and PrrA was determined by phosphorimaging.

Fig. 1 also shows that the higher levels of PrrB–P obtained in these experiments resulted in a concomitant increase in

phosphotransfer to PrrA, resulting in higher levels of PrrA–P in our phosphotransfer assays. This therefore confirms that subsequent signal transduction from PrrB to PrrA also occurs successfully under all conditions of DTT used under these in vitro conditions (Fig. 1). Interestingly, in the presence of 1 mM DTT (and to a lesser extent, 0.1 mM), PrrA–P levels were highest in the initial stages of phosphotransfer, with PrrA–P levels decreasing with time during the assays (Fig. 1B). PrrB possesses phosphatase as well as kinase activity towards PrrA [2,21], and these findings here may be attributable to higher phosphatase activity of PrrB under these partially reduced conditions. In confirmation with this, when phosphotransfer experiments were performed under the fully reduced conditions of 100 mM DTT, a sustained increase in PrrA–P levels is observed with time (Fig. 1).

When the irreversible disulfide reducing agent TCEP was used instead of DTT to generate reducing conditions, no detectable autophosphorylation occurred (Fig. 2). Preincubation of PrrB for several minutes in the presence of 1 mM DTT prior to the initiation of autophosphorylation assays by ATP addition, resulted in strong autophosphorylation of PrrB, as expected (Figs. 1 and 2A); however, when 2 mM TCEP was added just before ATP addition, detectable autophosphorylation was abolished (Fig. 2A). To determine if this inhibitory effect also occurs when DTT and TCEP are added into the assays at the same time, the assays were repeated but both reducing agents were added only just before initiation of the assays (with short preincubation occurring under oxidised, DTT-free conditions instead). Whilst control conditions in the absence of added reducing agent resulted in low but just detectable levels of phosphorylation as expected, the addition of 1 mM DTT and 2 mM TCEP together resulted in no detectable PrrB–P (Fig. 2B), confirming the inhibitory effect of TCEP on PrrB autophosphorylation. As positive control, 100 mM DTT addition was used; as expected, high levels of phosphorylation occurred, though the increase under these new assay conditions is not as great as that observed previously, presumably because of the different assay conditions employed (Fig. 2). Both DTT and TCEP are disulfide reductants; in recent years TCEP has been used as an alternative reducing agent to circumvent potential instability problems associated with DTT [30], but our results here indicate that it is not a suitable alternative for observing PrrB activity.

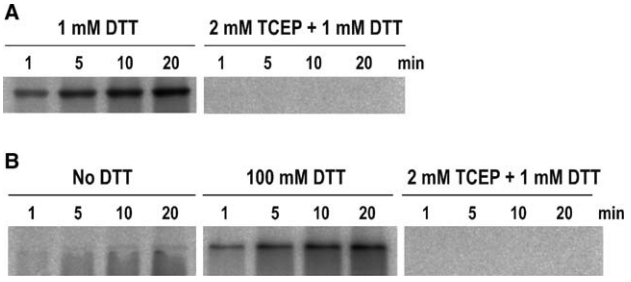

Fig. 2. Comparison of reducing agents ditheithreitol (DTT; a reversible thiol exchanger) and tris[carboxyethyl]phosphine (TCEP; an irreversible thiol agent) in modulation of PrrB autophosphorylation in vitro. (A) Reaction mixes (100 μl) were prepared as described [2] using 287 pmol PrrB and 1 mM DTT. Just prior to initiation of assays using 50 μM ATP and [γ-^{32}P]ATP as described [2], either TCEP was added to a final concentration of 2 mM, or no addition made. (B) Reaction mixes (100 μl) were prepared as above using 287 pmol PrrB in the absence of any added reducing agent. Just before initiation of assays using 50 μM ATP and [γ-^{32}P]ATP, either TCEP plus DTT were added to final concentrations of 2 and 1 mM, respectively, or 100 mM DTT alone, or no additions made, as positive and negative controls, respectively. Samples of 12.5 μl were removed to loading (stop) buffer, and resolved using 12% SDS–polyacrylamide gels. The quantity of ^{32}P associated with PrrB and PrrA was determined by phosphorimaging.

3.2. The effect of intracellular energy status on the signalling state of PrrB–P

The role of Prr, and of the homologous Reg system in *R. capsulatus*, in controlling the synthesis of a variety of cellular processes that produce or consume energy in the cell is well established. Whilst the roles of PrrB and Cco in redox sensing and signalling are well documented, there have been no in vitro studies that have specifically examined whether PrrB signalling might also be directly modulated by other intracellular signals relating to energy status and reducing power. To investigate this we examined the effects of ATP, ADP, AMP and NADH on maintenance of the PrrB signalling state, PrrB–P. Fig. 3 compares the effects of ATP, ADP and AMP on rates of PrrB–P dephosphorylation. When labelled PrrB–P was 'cold chased' with either excess cold ATP, excess cold ATP in the presence of an ATP-regenerative enzyme system or equimolar cold ATP + ADP, the only reaction in which PrrB–P levels were maintained was that containing the regenerative system i.e. in which no ADP was permitted to accumulate in the reactions (Fig. 3A). As control, the effect of each individual component of the regenerative system was tested individually, and only when the complete regenerative system was present was this effect observed (data not shown). Thus, no observable effects of ATP or AMP were observed. When NADH was used in similar experiments, levels of PrrB–P remained very similar to those of the control (data not shown). To examine the effects of ADP (and ATP, AMP) further and to test the direct effect of ADP in isolation, similar experiments were repeated in which PrrB–P was extensively washed prior to addition of either ATP, ADP or AMP individually. In experiments containing no additions, or added ATP or AMP (with a maintained regenerative system present), PrrB–P levels remained approximately constant. By contrast, in the presence of added ADP, dephosphorylation of PrrB–P was rapid (Fig. 3B), confirming the strong effect of ADP on PrrB–P dephosphorylation rate. The phosphorylation of any histidine protein kinase including PrrB is, in principal, a reversible reaction; presumably the accumulating ADP in these reactions drives the dephosphorylation of PrrB–P, and therefore this would account for the observed effects of ADP on PrrB–P levels shown here.

4. Discussion

Purified preparations of intact membrane protein PrrB respond to redox signals; increasing concentrations of the reversible thiol-disulfide exchanger DTT cause concomitant increases in phosphorylated PrrB levels, whilst air-oxidised PrrB barely phosphorylated at all (Fig. 1). This demonstrates the role of the intact protein as a primary sensor of redox signals. This is consistent with previous studies of intact cells which clearly show that conditions of redox potential and aerobiosis are linked to the presence of an intact Prr pathway [3,5]. The clear autophosphorylation response of isolated in-

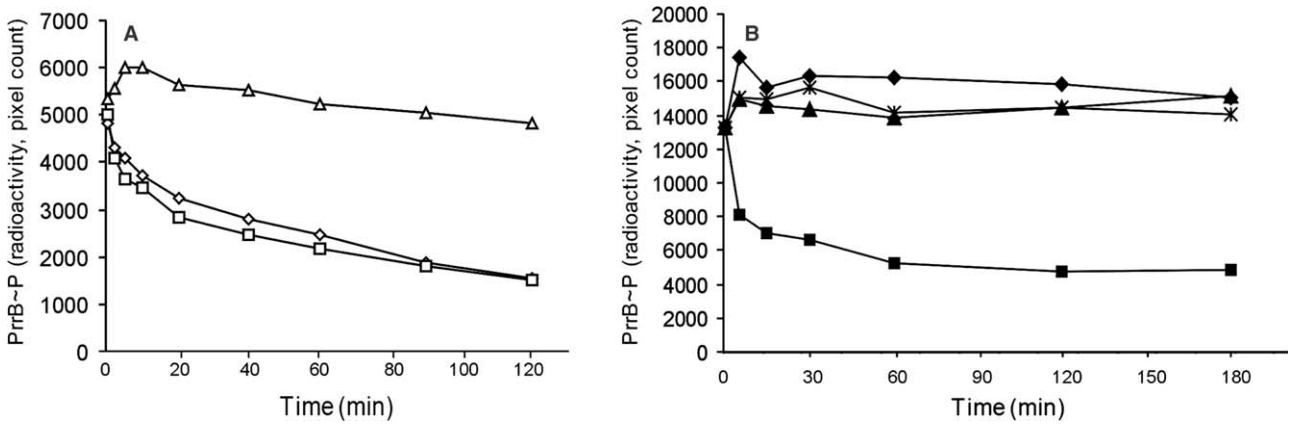

Fig. 3. Effect of ADP, ATP and AMP on the stability of PrrB–P. (A) The effect of an ATP regenerative system. About 287 pmol PrrB was allowed to autophosphorylate in the presence of [γ-^{33}P]ATP for 20 min in a 100 μl reaction volume as described [2]. 100-fold excess of cold ATP was then added to give a final concentration of 5 mM ATP in the presence (△) and absence (◇) of an ATP regenerative system (2 U pyruvate kinase and 100 nmol PEP), or 100-fold excess of each of ATP and ADP to give final concentrations of 5 mM ATP plus 5 mM ADP (□). At each time point, 2.9–7.0 pmol were removed to loading buffer and the quantity of ^{33}P associated with PrrB determined as described in Section 2. (B) PrrB (600 pmol) was allowed to autophosphorylate in the presence of [γ-^{33}P]ATP for 20 min in a 255 μl reaction mix containing 10 mM DTT as described in [2]. Residual ADP was converted to ATP by addition of 1 mM PEP and 2.5 U per 300 μl pyruvate kinase for 10 min. ATP, residual ADP and PEP were then removed by Centricon buffer exchange (4 × 200 μl washes with assay buffer). Protein was resuspended in 4 × 75 μl assay buffer aliquots containing either 100 μM ATP (♦), 100 μM ADP (■), 100 μM AMP (▲) or no additions ((×), control); ATP and AMP stock solutions were pre-treated with 1.25 U per 100 μl pyruvate kinase and 1 mM PEP prior to use, bringing the final amount of pyruvate kinase present per 300 μl reaction mix to 2.52 U and PEP to 13 μM. At each time point, 20 pmol were removed to loading buffer and the quantity of ^{33}P associated with PrrB determined as described in Section 2.

tact PrrB to increasing DTT-induced reducing conditions reported here contrasts with the findings of Swem et al. [23], who reported very low levels of autophosphorylation in response to reducing conditions using a truncated version of the *R. capsulatus* homologue RegB. The differences observed here may be attributable to the use in our studies of intact PrrB, rather than the truncated soluble domain. The presence or not of a transmembrane region is important for these studies, since it has been shown to play a role in PrrB signalling [2,21].

In addition to clear-cut responses to reducing conditions by intact PrrB in the assays, we also observed concomitant phosphotransfer to PrrA (Fig. 1). Taken together with previous studies [2] we confirm that our purified intact PrrB protein exhibits both signal sensing functions and phosphorylation responses. We also conclude there appears to be no requirement for added metal or other cofactors to observe these functions.

In the *R. capsulatus* homologue of PrrB, RegB, it was previously shown that a highly conserved cysteine residue located in the cytoplasm has a role in soluble RegB signal transduction in response to intracellular redox conditions [23]. Our results on the effects of DTT on intact PrrB autophosphorylation are consistent with the idea that a similar mechanism may also occur in this sensor too (Fig. 1). However, it is possible that other modulators may also contribute. The idea of multiple redox signal inputs into PrrB is consistent with the strict control that might be expected for this global regulator of gene expression, with multiple checkpoints for verifying the changes in redox potential prior to global changes in gene expression in *Rhodobacter*. Indeed, examples of multiple modulators of the signalling state of two-component systems have been reported previously, including electron transport quinones and proton motive force controlling the redox-responsive global Arc system in *E. coli* [24,25]. Indeed, a recent report suggested the involvement of ubiquinone as a signal for the closely-related PrrB homologue, RegB [32]. As in this study, these workers also used a full-length version of the sensor, but it was not clear whether the changes in autophosphorylation observed using oxidised and reduced ubiquinone/ubiquinol were attributable, at least in part, to the 10 mM DTT used to reduce the quinone [32].

In *E. coli*, the activity of redox sensor kinase ArcB was also shown to be modulated by levels of cytosolic metabolites such as D-lactate, acetate, pyruvate and NADH [26]. As some of these are indicators of cell energy status, it appears that energy status in addition to the identified redox signals (proton motive force, quinone electron carriers) also appears to be important. That at least one indicator of cell energy status affects Prr activity (and presumably that of other sensor kinases) was confirmed here (Fig. 3). ADP (but not ATP or AMP) exerted a significant decrease in levels of PrrB–P, but presumably this acts through reaction with PrrB–P resulting in PrrB and ATP products. Previous studies of purified NRII (a soluble sensor kinase) noted an effect of ADP on the extent of autophosphorylation [29,33]; NRII activity was consistently higher when ADP was removed from the reactions. In our studies of PrrB, we observed no increase in PrrB–P during autophosphorylation assays when ADP was removed (data not shown). The effects of ADP on the signalling state of PrrB demonstrated here (and presumably all other histidine protein kinases) may have implications for conditions of low cell energy in which ADP levels would be expected to rise; the significance of this in vivo has yet to be elucidated.

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